NUCLEIC ACID SEQUENCE AMPLIFICATION

Field of the Invention

This application is a continuation-in-part of Kacian et al., U.S. Serial Nos.: 07,855,732, filed March 19, 1992, 07/550,837, filed July 10, 1990, and 07/379,501, filed July
5   11, 1989, all hereby incorporated by reference herein.

This invention relates to methods for increasing the number of copies of a specific nucleic acid sequence or "target sequence" which may be present either alone or as a component, large or small, of a homogeneous or hetero-
10   geneous mixture of nucleic acids. The mixture of nucleic acids may be that found in a sample taken for diagnostic testing, environmental testing, for research studies, for the preparation of reagents or materials, for other processes such as cloning, or for other purposes.

15   The selective amplification of specific nucleic acid sequences is of value in increasing the sensitivity of diagnostic and environmental assays while maintaining specificity; increasing the sensitivity, convenience, accuracy and reliability of a variety of research
20   procedures; and providing ample supplies of specific oligonucleotides for various purposes.

The present invention is particularly suitable for use in environmental and diagnostic testing due to the convenience with which it may be practiced.

25   ## Background of the Invention

The detection and/or quantitation of specific nucleic acid sequences is an increasingly important technique for identifying and classifying microorganisms, diagnosing infectious diseases, detecting and characterizing genetic
30   abnormalities, identifying genetic changes associated with cancer, studying genetic susceptibility to disease, and measuring response to various types of treatment. Such

procedures have also found expanding uses in detecting and
quantitating microorganisms in foodstuffs, environmental
samples, seed stocks, and other types of material where the
presence of specific microorganisms may need to be

5   monitored. Other applications are found in the forensic
sciences, anthropology, archaeology, and biology where
measurement of the relatedness of nucleic acid sequences
has been used to identify criminal suspects, resolve
paternity disputes, construct genealogical and phylogenetic

10   trees, and aid in classifying a variety of life forms.

A common method for detecting and quantitating
specific nucleic acid sequences is nucleic acid hybrid-
ization. This method is based on the ability of two
nucleic acid strands that contain complementary or essen-

15   tially complementary sequences to specifically associate,
under appropriate conditions, to form a double-stranded
structure. To detect and/or quantitate a specific nucleic
acid sequence (known as the "target sequence"), a labelled
oligonucleotide (known as a "probe") is prepared that

20   contains sequences complementary to those of the target
sequence. The probe is mixed with a sample suspected of
containing the target sequence, and conditions suitable for
hybrid formation are created. The probe hybridizes to the
target sequence if it is present in the sample. The

25   probe-target hybrids are then separated from the single-
stranded probe in one of a variety of ways. The amount of
label associated with the hybrids is then measured as an
indication of the amount of target sequence in the sample.

The ,sensitivity of nucleic acid hybridization assays

30   is limited primarily by the specific activity of the probe,
the rate and extent of the hybridization reaction, the
performance of the method for separating hybridized and
unhybridized probe, and the sensitivity with which the

label can be detected. The most sensitive procedures may
lack many of the features required for routine clinical and
environmental testing such as speed, convenience, and
economy. Furthermore, their sensitivities may not be

5   sufficient for many desired applications.

As a result of the interactions among the various
components and component steps of this type of assay, there
is almost always an inverse relationship between
sensitivity and specificity. Thus, steps taken to increase

10   the sensitivity of the assay (such as increasing the
specific activity of the probe) may result in a higher
percentage of false positive test results. The linkage
between sensitivity and specificity has been a significant
barrier to improving the sensitivity of hybridization

15   assays. One solution to this problem would be to specific-
ally increase the amount of target sequence present using
an amplification procedure. Amplification of a unique
portion of the target sequence without amplification of a
significant portion of the information encoded in the

20   remaining sequences of the sample could give an increase in
sensitivity while at the same time not compromising
specificity.

A method for specifically amplifying nucleic acid
sequences termed the "polymerase chain reaction" or "PCR"

25   has been described by Mullis et al. (See U.S. patents
4,683,195, 4,683,202 and 4,800,159 and European patent
applications 86302298.4, 86302299.2, and 87300203.4 and
Methods in Enzymology, Volume 155, 1987, pp. 335-350.) The
procedure uses repeated cycles of primer dependent nucleic

30   acid synthesis occurring simultaneously using each strand
of a complementary sequence as a template. The sequence
that is amplified is defined by the locations of the primer
molecules that initiate synthesis. The primers are

complementary to the 3'-end portion of the target sequence
or its complement and must complex with those sites in
order for nucleic acid synthesis to begin. After extension
product synthesis, the strands are separated, generally by
5   thermal denaturation, before the next synthesis step. In
the PCR procedure, copies of both strands of a
complementary sequence are synthesized.

     The strand separation step used in PCR to separate the
newly synthesized strands at the conclusion of each cycle
10   of the PCR reaction is often thermal denaturation. As a
result, either a thermostable enzyme is required or new
enzyme must be added between thermal denaturation steps and
the initiation of the next cycle of DNA synthesis. The
requirement of repeated cycling of reaction temperature
15   between several different and extreme temperatures is a
disadvantage of the PCR procedure. In order to make the
PCR convenient, programmable thermal cycling instruments
are required.

     The PCR procedure has been coupled to RNA transcrip-
20   tion by incorporating a promoter sequence into one of the
primers used in the PCR reaction and then, after amplifi-
cation by the PCR procedure for several cycles, using the
double-stranded DNA as template for the transcription of
single-stranded RNA. (See, _e.g._, Murakawa _et al_., DNA
25   7:287-295 (1988).)

     Other methods for amplification of a specific nucleic
acid sequence comprise a series of primer hybridization,
extending and denaturing steps to provide an intermediate
double stranded DNA molecule containing a promoter sequence
30   through the use of a promoter sequence-containing primer.
The double stranded DNA is used to produce multiple RNA
copies of the target sequence. The resulting RNA copies
can be used as target sequences to produce further copies,

and multiple cycles can be performed. (See, e.g., Burg, et
al., WO 89/1050; Gingeras, et al., WO 88/10315 (sometimes
called "transcription amplification system" or TAS); EPO
Application No. 89313154 to Kacian and Fultz; EPO
5 Application No. 88113948.9 to Davey and Malek; Malek, et
al. WO91/02818.)

     Walker, et al., Proc. Natl. Acad. Sci. (USA) 89:392-
396 (Jan. 1992), not admitted to be prior art, describes an
oligonucleotide driven amplification method for use with a
10 DNA template, using a restriction endonuclease to produce
the initial target sequences and an enzyme to nick the
DNA/DNA complex in order to enable an extension reaction
and therefore amplification. Becker, et al., EPO
Application No. 88306717.5, describes an amplification
15 method in which a primer is hybridized to the target
sequence and the resulting duplex is cleaved prior to the
extension reaction and amplification; in the case where the
primer extends past the region of hybridization, it
requires cleavage prior to the extension and the primer
20 must be blocked at its 3'-end to prevent any unwanted
extension reactions from occurring prior to amplification.
Urdea, WO 91/10746, describes a signal amplification method
that incorporates a T7 promoter sequence.

     Other methods of amplifying nucleic acid include the
25 ligase chain reaction (LCR), described in European Patent
Application No. 320,308, in which at least four separate
oligoprobes are used; two of the oligoprobes hybridize to
opposite ends of the same target strand in appropriate
orientation such that the third and fourth oligoprobes may
30 hybridize with the first and second oligoprobes to form,
upon ligation, connected probes that can be denatured and
detected. Another method is that described in EPO
Application No. 0 427 073 A2, published May 15, 1991 and

not admitted to be prior art, in which a palindromic probe able to form a hairpin and having a functional promoter region in the hairpin is hybridized to a target sequence, then ligated to another oligonucleotide hybridized to the

5 target sequence such that specific RNA transcripts may be made.

Relatively large amounts of certain RNAs may be made using a recombinant single-stranded RNA molecule having a recognition sequence for the binding of an RNA-directed

10 polymerase, preferably $Q\beta$ replicase. (See, e.g., U.S. Patent No. 4,786,600 to Kramer, et al.) A number of steps are required to insert the specific sequence into a DNA copy of the variant molecule, clone it into an expression vector, transcribe it into RNA and then replicate it with

15 $Q\beta$ replicase.

Definitions

As used herein, the following terms have the following meanings unless expressly indicated to the contrary.

A.   Nucleic Acid.

20 "Nucleic acid" means either RNA or DNA, along with any nucleotide analogues or other molecules that may be present in the sequence and that do not prevent performance of the present invention.

B.   Template.

25 A "template" is a nucleic acid molecule that is able to be copied by a nucleic acid polymerase. A template may be either RNA or DNA, and may be any of single-stranded, double-stranded or partially double-stranded, depending on the polymerase. The synthesized copy is complementary to

30 the template. In this invention, the term copies also includes nucleic acid having the equivalent RNA or DNA

sequence to a template, which are commonly referred to as homologous sequences in the art.

     C.   Primer.

A "primer" is an oligonucleotide that is complementary
5   to a template that hybridizes with the template to give a primer/template complex for initiation of synthesis by a DNA polymerase, such as a reverse transcriptase, and which is extended by the addition of covalently bonded bases linked to its 3' end that are complementary to the
10   template. The result is a primer extension product. Virtually all DNA polymerases (including reverse tran-scriptases) that are known require complexing of an oligonucleotide to a single-stranded template ("priming") to initiate DNA synthesis. Under appropriate
15   circumstances, a primer may be a part of a promoter-primer. Such primers are generally between 10 and 100 bases in length, preferably between 20 and 50 bases in length.

     D.   Promoter or Promoter Sequence.

A "promoter" or "promoter sequence" is a specific
20   nucleic acid sequence that is recognized by a DNA-dependent RNA polymerase ("transcriptase") as a signal to bind to a nucleic acid molecule and begin the transcription of RNA at a specific site. For binding, such transcriptases generally require that the promoter and its complement be
25   double-stranded; the template portion need not be double-stranded. Individual DNA-dependent RNA polymerases recognize a variety of different promoter sequences that can vary markedly in their efficiency of promoting transcription. When an RNA polymerase binds to a promoter
30   sequence to initiate transcription, that promoter sequence is not part of the sequence transcribed. Thus, the RNA

transcripts produced thereby will not include the promoter sequence.

    E.   Promoter-primer.

    A promoter-primer comprises a promoter and a primer. It is an oligonucleotide that is sufficiently complementary to the 3'-end of a target nucleic acid sequence to complex at or near the 3'-end of that target nucleic acid sequence, which means that the promoter-primer complexes near enough the end of the target sequence to allow amplification of enough of the target sequence that the requirements of the assay, testing, cloning or other use for the amplified nucleic acid are met. The promoter-primer is used as a template to create a complementary nucleic acid sequence extending from the 3'-end (also known as the 3' terminus) of a target nucleic acid sequence, to result in a generally double stranded promoter, subject to any denaturing or enzymatic activity that may disrupt the double strand. Such promoter-primers are generally between 40 and 100 bases in length, preferably between 40 and 60 bases.

    A DNA- or RNA-dependent DNA polymerase also creates a complementary strand to the target nucleic acid molecule, using the target sequence as a template.

    F.   Modified Primer or Promoter-primer.

    The 3'-end of the primer or promoter-primer may be modified, or blocked, so as to prevent or reduce the rate and/or extent of an extension reaction from proceeding therefrom. A primer or promoter-primer having both modified and unmodified members consists of essentially the same nucleic acid sequence for the purposes of the present invention. In other words, the modified primer or promoter-primer does not contain a different complexing

sequence (primer) in that both the modified and unmodified oligonucleotide hybridize in effectively the same position (plus or minus about ten bases) on the target nucleic acid sequence. Also, the modified promoter-primer does not

5   contain a different recognition sequence (promoter) from the unmodified promoter-primer. This means that, within about 10 bases, the modified and unmodified primers or promoter-primers are the same, are recognized by the same RNA polymerase, and hybridize to more or less the same

10 target sequence (although not necessarily at precisely the same position). In a preferred embodiment, the modified and unmodified primers or promoter-primers are identical except for the modification.

        The 3'-end of the target complementary portion of a

15 primer or promoter-primer can be modified in a variety of ways well known to those skilled in the art. Appropriate modifications to a promoter-primer can include addition of ribonucleotides, 3' deoxynucleotide residues, (e.g., cordycepin (CO, Glen Research)), 3',2'-dideoxy nucleotide

20 residues, modified nucleotides with nonphosphodiester backbone linkages (such as phosphorothioates), and non-nucleotide linkages such as described in Arnold, et al., (PCT US 88/03173) (RS) or alkane-diol modifications (Wilk et al. Nuc. Acids Res. 18:2065, 1990) (RP), or the

25 modification may simply consist of one or more nucleotide residues 3' to the hybridizing sequence that are uncomplementary to the target nucleic acid. Of course, other effective modifications are possible as well.

        A mixture of modified and unmodified oligonucleotides

30 may be used in an amplification reaction, and a broad range of ratios of modified to unmodified oligonucleotide (e.g., from 1:1 to 1,000:1) can be used. A mixture of

oligonucleotides with different 3′ modifications may also be used.

G.   Plus (+) and Minus (-) Strand(s).

Discussions of nucleic acid synthesis are greatly
5   simplified and clarified by adopting terms to name the two complementary strands of a nucleic acid duplex. Tradition- ally, the strand encoding the sequences used to produce proteins or structural RNAs was designated as the "plus" strand and its complement the "minus" strand. It is now
10   known that in many cases, both strands are functional, and the assignment of the designation "plus" to one and "minus" to the other must then be arbitrary. Nevertheless, the terms are very useful for designating the sequence orientation of nucleic acids and will be employed herein
15   for that purpose, with the "plus" strand denominating the original target sequence strand that is complexed with the first primer or promoter-primer.

H.   Target Nucleic Acid Sequence, Target Sequence.

A "target nucleic acid sequence," or "target
20   sequence," has a desired nucleic acid sequence to be amplified, and may be either single-stranded or double- stranded and may include other sequences 5′ or 3′ of the sequences to be amplified which may or may not be amplified.
25   The target nucleic acid sequence includes the complex- ing sequences to which the promoter-primer hybridizes during performance of the present invention. Where the target nucleic acid sequence is originally single-stranded, the term refers to either the (+) or (-) strand, and will
30   also refer to the sequence complementary to the target sequence. Where the target nucleic acid sequence is

originally double-stranded, the term refers to both the (+) and (-) strands.

   I.   <u>DNA-Dependent DNA Polymerase</u>.

   A "DNA-dependent DNA polymerase" is an enzyme that
5 synthesizes a complementary DNA copy from a DNA template.
An example is bacteriophage T7 DNA polymerase. All known
DNA-dependent DNA polymerases require a complementary
primer, which can be RNA or DNA, or a copolymer, to
initiate synthesis. It is known that under suitable
10 conditions certain DNA-dependent DNA polymerases may syn-
thesize a complementary DNA copy from an RNA template.

   J.   <u>DNA-Dependent RNA Polymerase (Transcriptase)</u>.

   A "DNA-dependent RNA polymerase" or "transcriptase" is
an enzyme that synthesizes multiple RNA copies from a
15 double-stranded or partially-double stranded DNA molecule
having a (usually double-stranded) promoter sequence. It
should be noted that the present invention includes single
stranded promoter sequences in the promoter-primer, along
with the RNA polymerases that recognize them. The RNA
20 molecules ("transcripts") are synthesized in the 5' → 3'
direction of the RNA molecule, beginning at a specific
position just downstream of the promoter. Examples of
transcriptases are the DNA-dependent RNA polymerases from
bacteriophages T7, T3, and SP6.

K.   <u>RNA-Dependent DNA Polymerase (Reverse Transcriptase)</u>.

An "RNA-dependent DNA polymerase" or "reverse tran-scriptase" is an enzyme that synthesizes a complementary

5   DNA copy from an RNA template. All known reverse tran-scriptases also have the ability to make a complementary DNA copy from a DNA template; thus, they are both RNA- and DNA-dependent DNA polymerases. A primer is required to initiate synthesis with either the RNA or DNA templates.

10   L.   <u>RNAse H</u>.

An "RNAse H" is an enzyme that degrades the RNA portion of an RNA:DNA duplex. RNAse H's may be endo-nucleases or exonucleases. Avian myeloblastosis virus and Moloney murine leukemia virus reverse transcriptases

15   contain an RNAse H activity in addition to their polymerase activity. Some cloned reverse transcriptases lack RNAse H activity. There are also sources of RNAse H available without an associated polymerase activity. The degradation may result in separation of RNA from an RNA:DNA complex.

20   Alternatively, the RNAse H may simply cut the RNA at various locations such that portions of the RNA melt off or permit enzymes to unwind portions of the RNA, or the RNA fragments generated may serve as primers for extension by a polymerase.

25   M.   <u>Hybridize, Complex</u>.

The terms "hybridize" and "complex" refer to the formation of duplexes between nucleotide sequences that are sufficiently complementary to form duplexes (or "com-plexes") via Watson-Crick base pairing. Where a promoter-

30   primer or primer "hybridizes" with target (template), such complexes (or hybrids) are sufficiently stable to serve the

priming function required by a DNA polymerase to initiate
DNA synthesis.

N.   Specificity

Specificity is a characteristic of a nucleic acid
5   sequence that describes its ability to distinguish between
target and non-target sequences, dependent on sequence and
assay conditions.

Summary of the Invention

The present invention is directed to a novel,
10   autocatalytic method of synthesizing multiple copies of a
target nucleic acid sequence (i.e., the method cycles
automatically without the need to modify reaction
conditions such as temperature, pH, or ionic strength).

The present invention features treating a target
15   sequence with a first oligonucleotide (that has a
complexing sequence sufficiently complementary to a 3'-end
portion of the target sequence to hybridize therewith (this
alone is termed a primer), and that has a sequence 5' to
the complexing sequence that includes a sequence which, in
20   double-stranded form, acts as a promoter for an RNA
polymerase (this arrangement is termed a promoter-primer)),
and a second oligonucleotide (which is a primer or
promoter-primer that has a complexing sequence sufficiently
complementary to the complement of the target sequence to
25   hybridize therewith), under conditions in which an
oligonucleotide/target sequence complex may be formed and
DNA and RNA synthesis may occur. In this invention, one or
both of the first and second oligonucleotides is a mixture
of a blocked and an unblocked oligonucleotide sequence
30   (blocked oligonucleotides have a modified 3' end to prevent
or reduce the rate and/or extent of primer extension by a
DNA polymerase), or a mixture of oligonucleotides with

different 3' modifications. Such a mixture significantly enhances the efficiency of the specific amplification reaction compared to use of only blocked or only unblocked oligonucleotides. The ratio of such oligonucleotides can

5   be varied dependent upon the specific template sequence to be amplified, but generally is between 1:1 and 1000:1 blocked to unblocked. The invention does not require that the target sequence have defined 3'- or 5'-ends.

One aspect of the invention includes (a) treating a

10 target sequence with a first promoter-primer oligonucleotide that has a complexing sequence sufficiently complementary to a 3'-end portion of the target sequence to hybridize therewith, and that has a sequence 5' to the complexing sequence that includes a sequence which, in

15 double-stranded form, acts as a promoter for an RNA polymerase, under conditions in which an oligonucleotide/target sequence complex may be formed and DNA synthesis may be initiated by an appropriate polymerase (e.g., a DNA polymerase), (b) incubating the first

20 oligonucleotide/target complex under extension reaction conditions so that the 3'-end of the target may be extended to produce a hybrid template for an RNA polymerase; and (c) incubating the hybrid template under conditions in which multiple RNA copies of the target sequence may be produced

25 using an RNA polymerase that recognizes the promoter sequence. The invention also includes generation of a 3'-end of an RNA target sequence in step (b) by the action of an enzyme that selectively degrades the RNA portion of an RNA:DNA hybrid (e.g., RNase H). The RNA so produced may

30 autocatalytically cycle to produce more product.

In other methods, the invention features (a) contacting a nucleic acid (e.g., RNA or DNA) target sequence with a first oligonucleotide primer or promoter-

primer under conditions in which a first oligonucleotide/target sequence complex is formed such that DNA synthesis may be initiated by an appropriate polymerase (e.g., a DNA polymerase), (b) incubating the first

5 oligonucleotide under extension reaction conditions so that the target may be used by the polymerase as a template to give a first DNA extension product complementary to the target (if the first primer is not blocked); (c) if the target is an RNA molecule, separating the DNA extension

10 product from the RNA target using an enzyme that selectively degrades the RNA target, or if the target is a DNA molecule, separating the two DNA strands (e.g., by heating at 90-100°C, or by other means); (d) contacting the DNA extension product with a second oligonucleotide that

15 includes a primer or a promoter-primer, and that has a complexing sequence sufficiently complementary to the 3'-end portion of the DNA extension product to hybridize therewith under conditions in which a second oligonucleotide/extension product complex is formed and DNA

20 synthesis may be initiated as above, depending on any blocking molecules on this primer. In this invention, if the first oligonucleotide is not a promoter-primer, then the second oligonucleotide is a promoter-primer, which means the second oligonucleotide has a sequence 5' to the

25 complexing sequence that includes a promoter sequence for an RNA polymerase. In addition, the first and/or second oligonucleotides consist of either a mixture of a blocked and an unblocked oligonucleotide, or a mixture of oligonucleotides with different 3' modifications.

30   The amplification reaction is performed in a mixture consisting essentially of the necessary reactants and reagents. However, such a mixture may also contain enzymes or other substituents that do not qualitatively affect the

amplification of the invention (e.g., the mechanism of the reaction). Such substituents may affect the amount of amplification observed. For example, the mixture may contain other promoter-primers for the same target

5   sequence, or may contain "helper" oligonucleotides. Such helper oligonucleotides are used in a manner similar to the hybridization helper probes described by Hogan et al., U.S. Patent 5,030,557 (hereby incorporated by reference herein), namely by aiding binding of the promoter-primer to its

10 target nucleic acid, even if that target nucleic acid has significant secondary structure. Despite the similarity in use of such helper oligonucleotides, it was surprising that such helper oligonucleotides could be used in an amplification protocol without adverse effect on the

15 efficiency of the procedure.

    The first oligonucleotide may be a promoter-primer and the second oligonucleotide may be a primer, or vice versa, or both the first and second oligonucleotides may be promoter-primers, with either identical promoters (in the

20 sense that the promoters are recognized by the same RNA polymerase) or different promoters. Use of different promoters is particularly useful when the amplified nucleic acid will be used for cloning. The first and second oligo-nucleotides and the RNA produced from the target sequence

25 may then be used to autocatalytically synthesize multiple copies (by which is meant both complementary and homologous nucleic acid sequences) of the target sequence.

    The modified primer or promoter-primer of the present invention consists essentially of a single nucleic acid

30 sequence that has a modification at or near (within 3 bases) the 3'-end of the given primer or promoter-primer that alters (decreases or blocks) extension of the primer on a template by a DNA polymerase. Preferably this

modified primer or promoter-primer is mixed with an unmodified primer or promoter-primer consisting essentially of the same nucleic acid sequence, along with one or more other primers or promoter-primers of a different nucleic acid sequence (that may also be a mixture of blocked and unblocked oligonucleotides). The invention also includes use of mixtures of primers and promoter-primers with more than one modification at or near their 3'-ends.

In addition, in another aspect of the present invention, where the sequence sought to be amplified is DNA, use of an appropriate preliminary procedure may enhance generation of RNA copies that may then be amplified according to the present invention. Accordingly, the present invention is also directed to preliminary procedures for use in conjunction with the amplification method of the present invention that not only can increase the number of copies to be amplified, but also can provide RNA copies of a DNA sequence for amplification.

In a further aspect, the invention features generation of a defined 5' end (i.e., one of known sequence) in an RNA target sequence by treating the RNA with a DNA oligonucleotide which hybridizes near the second primer binding site and thereby forms a substrate for RNAse H. This substrate is then cleaved by RNAse H to define the 5' end of the RNA target, which can be amplified as discussed above.

In another aspect, the present invention involves cooperative action of a DNA polymerase (such as a reverse transcriptase) and a DNA-dependent RNA polymerase (tran- scriptase) with an enzymatic hybrid-separation step to produce products that may themselves be used to produce additional product, thus resulting in an autocatalytic reaction without requiring manipulation of reaction

conditions, such as in thermal cycling. Further, in some
embodiments of the present invention that include a prelim-
inary procedure, all but the initial step(s) of the
preliminary procedure are carried out at one temperature.

5   The present invention may be used as a component of
assays to detect and/or quantitate specific nucleic acid
target sequences in clinical, environmental, forensic, and
similar samples or to produce large numbers of copies of
DNA and/or RNA of a specific target sequence for a variety

10 of uses. These methods may also be used to produce
multiple DNA copies of a DNA target for cloning, or to
generate probes, or to produce RNA and DNA copies for
sequencing.

       In one example of a typical assay, a sample (including

15 RNA or DNA target) to be amplified is mixed with a buffer
concentrate containing the buffer, salts (e.g., divalent
cations such as magnesium), nucleotide triphosphates,
primers and/or promoter-primers (blocked and/or unblocked),
a thiol reducing agent such as dithiothreitol, and a

20 polycation such as spermidine. The reaction is then
optionally incubated near 100°C to denature any secondary
structure. After cooling to room temperature (about 20°C),
enzymes containing DNA and RNA dependent DNA polymerase
activity, RNAse H activity and DNA dependent RNA polymerase

25 activity are added and the mixture is incubated for about
10 minutes to four hours at 37°C to 42°C. The reaction can
then be assayed by adding a luminescently-labelled probe,
incubating 10 to 30 minutes at 60°C, adding a solution to
selectively hydrolyze the label on unhybridized probe,

30 incubating the reaction for 5 to 10 minutes at 60°C, and
measuring the remaining chemiluminescence in a luminometer.
Ins. E2 (See, e.g., Arnold, et al., ~~PCT US88/03246 (filed September~~
E   ~~21, 1988, published March 23, 1989)~~ the disclosure of which

is incorporated herein by reference and is referred to as "HPA".) The products of the invention may be used in many other assay systems known to those skilled in the art.

Optionally, a DNA target without a defined 3'-end, can
5 be incubated near 100°C to denature any secondary structure and cooled to room temperature. Reverse transcriptase is added and the reaction mixture is incubated for 12 minutes at 42°C. The reaction is again denatured near 100°C, this time to separate the primer extension product from the DNA
10 template. After cooling, enzymes with DNA and RNA dependent DNA polymerase activity, RNAse H activity and DNA dependent RNA polymerase are added and the reaction is incubated for 10 minutes to four hours at 37°C-42°C. For a DNA target, a defined 3'-end can be created by use of a
15 restriction endonuclease. A defined 3'-end may also be generated by other means known in the art.

Yet another aspect of the invention features a composition consisting essentially of a first and a second oligonucleotide of opposite sense and able to hybridize at
20 or near the 3'-end of a target nucleic acid sequence and its complement, respectively, wherein one of the oligonucleotides is a promoter-primer and the other may be either a primer or a promoter-primer, and one or both of the oligonucleotides consists essentially of a mixture of a
25 single nucleic acid sequence having either a modified or an unmodified 3'-end, a DNA-dependent DNA polymerase, an RNA-dependent DNA polymerase, and an RNA polymerase, wherein the mixture allows amplification at effectively constant pH, concentration and temperature (i.e., none of the
30 recited conditions need be actively changed by the user). The composition may also include an RNAse H activity and/or other components described herein.

In other aspects, the invention features kits
containing oligonucleotides including specific sequences
useful in this amplification method, or in other
amplification methods, such as those described above. Such
5 sequences include those listed in the SEQUENCE LISTING, and
may be attached to other sequences recognized by an enzyme
(such as a polymerase, or restriction endonuclease). In
particular, these oligonucleotides are useful for
amplifying Mycobacterium nucleic acid, e.g., that of M.
10 tuberculosis, and may have modified 3'-ends as discussed
above.

The materials used in the present invention may be
incorporated as part of diagnostic kits or other kits for
use in diagnostic procedures, or other procedures, and the
15 invention is adaptable to multi-well technology which may
be provided in kit format.

Brief Description of the Drawings

Figure 1 shows the structure of the alkane-diol
modification referred to as RP.

20   Detailed Description of the Invention

In accordance with the present invention, a novel
method, composition and kit are provided for the amplifica-
tion of specific nucleic acid target sequences for use in
assays for the detection and/or quantitation of specific
25 nucleic acid target sequences or for the production of
large numbers of copies of DNA and/or RNA of specific
target sequences for a variety of uses.

The present invention advantageously provides an
amplification method that synthesizes RNA copies of a
30 target sequence by use of a mixture of blocked and

unblocked promoter-primers, or promoter-primers with
different 3' modifications, consisting essentially of the
same nucleic acid sequence in a ratio that provides for
lessened non-specific byproducts. In the present
5   invention, the amplification process occurs spontaneously
and isothermally under a broad range of conditions. The
amplification reactions described below are a series of
logical steps. The relative rate of each step will
determine the effective yield of amplification product.
10 Use of a mixture of blocked and unblocked primers reduces
the side reactions, and hence improves amplification. Side
products, such as "primer-dimers" have been described, and
are well known in the art to affect the efficiency of
amplification reactions. The present invention reduces the
15 efficiency of formation of such byproducts, therefore
enhancing amplification efficiency.

Suitable DNA polymerases for the present invention
include reverse transcriptases such as avian myeloblastosis
virus (AMV) reverse transcriptase and Moloney murine
20 leukemia virus (MMLV) reverse transcriptase. Promoters or
promoter sequences suitable for incorporation in promoter-
primers used in the present invention are nucleic acid
sequences (either naturally occurring, produced synthetic-
ally or by a restriction endonuclease digest) that are
25 specifically recognized by an RNA polymerase that recog-
nizes and binds to that sequence and initiates the process
of transcription whereby RNA transcripts are produced.
Promoter sequences for which there is a known and available
polymerase that is capable of recognizing the initiation
30 sequence are particularly suitable to be employed. Such
promoters include those that are recognized by certain
bacteriophage polymerases such as those from bacteriophage
T3, T7 or SP6. The sequence may optionally include

nucleotide bases extending beyond the actual recognition
site for the RNA polymerase that may impart added stability
or susceptibility to degradation processes or increased
transcription efficiency.

5   Although some of the reverse transcriptases suitable
for use in the present invention have an RNAse H activity,
such as AMV or MMLV reverse transcriptase, it may be pre-
ferred to add exogenous RNAse H, such as E. coli RNAse H.
For example, although the Examples (see below) show that
10   the addition of exogenous RNAse H is not required, the
RNAse H activity present in AMV reverse transcriptase may
be inhibited by relatively large amounts of heterologous
DNA present in the reaction mixture; one solution to the
problem is to add exogenous RNAse H.   Another instance when
15   added RNAse H may be required is when an oligonucleotide
hybridizes internally (i.e., the oligonucleotide hybridizes
such that target sequence nucleotides extend past both the
3′ and 5′ ends of the oligonucleotide) on the target RNA.
        The present invention does not require a denaturation
20   step to separate the RNA-DNA complex produced by the first
DNA extension reaction.   Such denaturation steps require
manipulation of reaction conditions such as by substan-
tially increasing the temperature of the reaction mixture
(generally from ambient temperature to about 80°C to about
25   105°C), reducing its ionic strength (generally by 10X or
more) or changing pH (usually increasing pH to 10 or more).
Such manipulations of the reaction conditions often
deleteriously affect enzyme activities, requiring addition
of additional enzyme and also necessitate further manipula-
30   tions of the reaction mixture to return it to conditions
suitable for further nucleic acid synthesis.
        The second oligonucleotide in the mixture may be
blocked or modified similarly to the first oligonucleotide.

In one aspect of the present invention, if the first
oligonucleotide is unmodified, then the second
oligonucleotide is modified. Also, if the first oligo-
nucleotide is not a promoter-primer, then the second oligo-
5 nucleotide is a promoter-primer. Further, if the first
oligonucleotide is only a primer, then it may be unblocked,
and the second oligonucleotide is then a promoter-primer
including both blocked and unblocked constituents
consisting essentially of a single nucleic acid sequence.
10   Surprisingly, such a mixture of blocked and unblocked
oligonucleotides consisting essentially of the same nucleic
acid sequence reduces the amount of non-specific product
formation, and thereby increases the effectiveness of the
amplification.
15   The RNA copies or transcripts produced may auto-
catalytically multiply without further manipulation.
        In another aspect of the present invention, the first
and second oligonucleotides are both promoter-primers, and
either or both may each consist of both modified and unmod-
20 ified promoter-primers. In such a case, it is preferred
that both promoters are recognized by the same RNA
polymerase unless it is intended to introduce the second
promoter for purposes other than amplification, such as
cloning. Where both oligonucleotides are promoter-primers,
25 then transcripts complementary to both strands of the
double-stranded template will be produced during the
autocatalytic reaction and the number of copies of the
target sequence synthesized may be enhanced.
        Note that, as the first oligonucleotide (primer or
30 promoter-primer) defines one end of the target sequence,
the second oligonucleotide (primer or promoter-primer) now
defines the other end; the termini may also be defined by a
specific restriction endonuclease, or by other suitable

means (which may include a natural 3'-end). The RNA tran-
scripts may have different termini from the original target
nucleic acid, but the sequence between the first
oligonucleotide and the second oligonucleotide remains

5   intact. The RNA transcripts so produced may automatically
recycle in the above system without further manipulation.
Thus, this reaction is autocatalytic.

Also note that either oligonucleotide may have
nucleotide sequences 5' to its priming sequence that can

10   result in the addition of extra nucleotide sequence to the
eventually resulting double stranded DNA; the extra
nucleotide sequence is not limited to a promoter sequence.

In another embodiment, the present invention may
consist of a first and second oligonucleotide in which a

15   promoter-primer is provided which consists only of a
blocked oligonucleotide, or only of an unblocked
oligonucleotide, or an oligonucleotide with a mixture of
different modifications at or near the 3'-end.

In further embodiments, the amplification is performed

20   in the presence of additives to enhance amplification.
Examples such as dimethyl sulfoxide, dimethyl formamide,
ethylene glycol, glycerol or zinc have been used.

The components of the reaction mixture may be added
stepwise or at once. The reaction advantageously takes

25   place under conditions suitable for maintaining the
stability of reaction components, such as the component
enzymes, and without requiring modification or manipulation
of reaction conditions during the course of the
amplification reaction.

30   The present invention may be used as a component of
assays to detect and/or quantitate specific nucleic acid
target sequences in clinical, environmental, forensic, and
similar samples or to produce large number of copies of DNA

and/or RNA of specific target sequence for a variety of
uses.

Examples

Preface

5   The following examples demonstrate the utility of the
methods of the present invention. They are not limiting
and should not be considered as such.

       Unless otherwise specified the reaction conditions for
amplification used in the following examples were 50 mM
10   Tris-HCl, 35 mM KCl, 20 mM $MgCl_2$, 15 mM N-acetyl-cysteine,
4 mM rATP, 4 mM rCTP, 4 mM rGTP, 4 mM rUTP, 1 mM dATP, 1 mM
dCTP, 1 mM dGTP, 1 mM dTTP, 10% glycerol, 10% dimethyl
sulfoxide, 300-600 units MMLV reverse transcriptase, 200-
400 units T7 RNA polymerase, 0.15 μM each primer or
15   promoter-primer, and specified amounts of template and
enzymes in 100 μl volumes at 42°C for one hour.
Dithiothreitol, spermidine and/or polyethyleneimine (PEI)
may also advantageously be added to the reaction mixture.

       The enzymes used in the following examples are T7 or
20   T3 RNA polymerase and Moloney murine leukemia virus (MMLV)
reverse transcriptase. Other RNA polymerases with
different promoter specificities are also suitable.

       The relative amplification was measured as follows. A
sample of the amplification reaction mixture (usually 10
25   μl) was added to 100 μl of a luminescently labelled probe
(for example, labelled with an acridinium ester - see HPA
reference above) solution containing approximately 75 fmol
probe, 0.1 M lithium succinate, pH 4.7, 2% (w/v) lithium
lauryl sulfate, 15 mM aldrithiol, 20 mM EDTA, and 20 mM
30   EGTA, and mixed. The reactions were then incubated 20
minutes at 60°C and cooled. To each hybridization reaction

was added 300 μl of 0.6 M sodium borate pH 8.5, 1% Triton
X-100.   The reactions were then mixed and incubated six
minutes at 60°C to destroy the chemiluminescent label of
the unhybridized probe. This method of destruction of the
5 chemiluminescent label of unhybridized probe is quite
specific; only a very small fraction of the unhybridized
probe remains chemiluminescent.   The reactions were cooled
and the remaining chemiluminescence was quantified in a
luminometer upon the addition of 200 μl 0.1% hydrogen
10 peroxide, 1 mM nitric acid, and surfactant, and 200 μl 1.0
N sodium hydroxide.   In the assay, hybridized probe emits
light. The quantity of photons emitted are measured in a
luminometer and the results are reported as Relative Light
Units or RLU.   Since the reaction that destroys the
15 chemiluminescent label of unhybridized probe is not 100%
effective, there is generally a background level of signal
present in the range of about 1000 to 2000 RLU.

Many other assay methods are also applicable,
including assays employing hybridization to isotopically
20 labeled probes, blotting techniques and electrophoresis.

These reaction conditions are not necessarily
optimized, and have been changed as noted for some systems.
The oligonucleotide sequences used are exemplary and are
not meant to be limiting as other sequences have been
25 employed for these and other target sequences.

Example 1

To show that amplification occurred with a modified
promoter-primer complementary to a sequence within an RNA
target, a promoter-primer complementary to a sequence
30 within M. tuberculosis rRNA (Seq ID No. 1) was synthesized
either unmodified or with a 3' alkane diol (RP) or 3'
cordycepin (CO) and incubated with a primer of the same

sense as the target RNA (Seq ID No. 2) and 3 tmol of target
under the conditions described above. The reactions were
analyzed with a probe of the same sense as the target RNA
(Seq ID No. 3) with helper oligonucleotides as described in
Hogan (U.S. Patent 5,030,557, Means for Enhancing Nucleic
Acid Hybridization, Seq ID Nos. 4 and 5). The results show
that significant amplification does occur with a promoter-
primer containing a 3' modification.

Promoter-primer modification	RLU
Unmodified	314,445
3'cordycepin	71,382
Unmodified	683,737
3'-RP	70,014

Example 2.

In this experiment, a promoter-primer with a sequence
complementary to M. tuberculosis 23S rRNA was modified by
the presence of a 3' phosphorothioate nucleotide. Fifteen
pmol of promoter-primer and primer (Seq ID Nos. 6 and 7)
were used to amplify 0.3 tmol of target RNA, followed by
detection with probe the same sense as the target RNA (Seq
ID No. 8) with helper probes (Seq. 10 Nos. 9 and 10). The
results show that 3' phosphorothioate modified promoter-
primer worked as well as unmodified oligonucleotide.

Promoter-primer	RLU + target	RLU - target
Unmodified	2,614,079	899
3' phosphorothioate	2,570,798	647

Example 3.

       To show that mixtures of modified and unmodified
promoter-primers function in an amplification assay,
reactions were performed with 15 pmol of the primer and a
 5 promoter-primer (see below) and assayed as described in
Example 1.   Three tmol of target RNA was used.

		Pmol Promoter-primer		
		Unmodified	CO-modified	RLU
Experiment 1	+Target	15	0	834,902
10	+Target	3	12	971,938
	-Target	3	12	1,456
Experiment 2	+Target	3	12	1,015,199
	+Target	0.1	15	961,041

       The results show that a mixture of blocked and
15 unblocked oligonucleotides worked as well or better than
all unblocked even at a ratio of 1:150 unblocked to
blocked.

Example 4.

       In this experiment 3 tmol of target RNA were incubated
20 with different concentrations of CO blocked and unblocked
primer and a mixture of 15 pmol CO blocked promoter-primer
and 0.1 pmol unblocked promoter-primer as in Example 1.
Products were detected by hybridization assay.

	Pmol Primer		RLU
	Blocked	Unblocked	
	0	15	969,522
	10	5	802,840
	13	2	648,271

       In addition to the satisfactory amplification
30 observed, it was surprisingly found that the amount of non-

template directed product was significantly less in the reactions performed with blocked oligonucleotides compared to reactions performed with unblocked oligonucleotides.

Example 5.

In this experiment, the effect of mixing a single oligonucleotide sequence with two different 3' modifications was demonstrated. Three tmol of target RNA was amplified as in Example 1. The promoter-primer was synthesized with an unblocked 3'-end, blocked with RP, or CO blocked. Two pmol of primer were used.

Pmol Promoter-primer			RLU
RP modified	CO modified	Unmodified	
0	15	0.1	450,157
2	13	0.01	681,647
2	13	0	678,871
5	10	0	755,839

This example shows that a mixture of unmodified and modified or a mixture of different types of modified promoter-primers amplified well, allowing detection of 3 tmol of RNA target in one hour.

Example 6.

In this example, a mixture of modified and unmodified primers and promoter-primers were used to amplify 3 tmol M. tuberculosis rRNA. A mixture of 2 pmol RP-modified promoter-primer and 13 pmol of CO-modified promoter-primer were incubated with unmodified primer or a mixture of unmodified primer and primer synthesized with a 3' phosphorothioate nucleotide (PS). The sequences and hybridization probes are as in Example 1.

Primer modification		RLU
Unmodified	PS modified	
--	15 pmol	118,411
1 pmol	14 pmol	364,733
5 No target		1,266

Under these conditions, the mixture of modified and unmodified primers work best.

Example 7.

In this example, 80 tmol of <u>Neisseria gonorrhoeae</u> rRNA
10 was amplified with a primer complementary to the rRNA (Seq. I.D. No. 13) and a mixture of 28 pmol 3'-RP blocked- and 2 pmol unblocked promoter primer of the same sense as the RNA target (Seq. I.D. No. 14). In some reactions, a 3'-blocked oligonucleotide (Seq. I.D. No. 15) capable of hybridizing
15 to <u>N</u>. <u>gonorrhoeae</u> rRNA and forming an RNAse H substrate, was added to the amplification. An aliquot of the reactions was hybridized to an AE-labeled probe and two helper probes complementary to the rRNA sequence (Seq. I.D. Nos. 16, 17, and 18, respectively).

20 RLU - RNAse H substrate oligo RLU + RNAse H substrate oligo

7,910	32,473
16,337	728,246
17,304	80,487
12,518	51,893

25 Example 8.

In this example, 3 or 30 tmol of <u>M</u>. <u>tuberculosis</u> rRNA was amplified with a primer (Seq. I.D. No. 7) and a mixture of 14 pmole of 3'-RP blocked- and 1 pmol unblocked promoter primer containing a promoter for T3 RNA polymerase (Seq.
30 I.D. No. 19). The reaction was performed as in Example 1 except that 450 units of MMLV RT were used, 200 units of T3

RNA polymerase replaced the T7 RNA polymerase, and the reaction was terminated after 40 minutes.

Target concentration	RLU value
30 tmol	358,053
3 tmol	75,440
0 tmol	553

The results demonstrate that a mixture of blocked and unblocked promoter primer can be used to amplify RNA using reverse transcriptase and T3 RNA polymerase.

Example 9.

In this example, amplification of a DNA target with an RP modified promoter primer was examined. Three tmol of cloned HIV-1 DNA was incubated with 30 pmol of a primer with sequence 5'- ATAATCCACCTATCCCAGTAGGAGAAAT-3' (SEQ. ID. NO. 20) and a promoter primer with sequence 5'- AATTTAATACGACTCACTATAGGGAGACCACACCTTGTCTTATGTCCAGAATGCT-3' (SEQ. ID. NO. 21) at 95°C for 5 minutes, then cooled to room temperature. After enzyme addition, the reaction was incubated at 37°C for 2 hours. The conditions were 50 mM Tris-HCl, 40 mM potassium acetate pH 8, 18 mM $MgCl_2$, 5 mM DTT, 2 mM spermidine, 6.2 mM GTP, 6.2 mM ATP, 2 mM CTP, 2 mM UTP, 0.2 mM dTTP, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dCTP, 800 U MMLV RT, 400 U T7 RNA polymerase. The promoter primer was unmodified or modified with an RP at the 3' end. The reactions were assayed with AE-labeled probe of the same sense as the primer. Results shown are the average of five replicates.

Pmol promoter primer		
Unmodified	Modified	Average RLU
30	0	127,223
26	4	411,692
0	30	743,877

It was unanticipated and surprising that amplification of a DNA target, especially one without a defined 3'-end, was not inhibited by the use of a modified promoter primer.

5   The present embodiments of this invention are to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims therefore are intended
10   to be embraced therein.